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Protection against soman or VX poisoning by human butyrylcholinesterase in guinea pigs and cynomolgus monkeys

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Abstract

Human butyrylcholinesterase (HuBuChE), purified from outdated human plasma, is being evaluated for efficacy against nerve agents in guinea pigs and cynomolgus monkeys. Previous studies in rodents and nonhuman primates demonstrated that pretreatment of animals with enzymes that can scavenge nerve agents could provide significant protection against behavioral and lethal effects of nerve agent intoxication. In preparation for evaluation of efficacy of HuBuChE prior to initiating an investigational new drug (IND) application, the pharmacokinetics of HuBuChE were evaluated in guinea pigs and in cynomolgus monkeys. HuBuChE was injected intramuscularly (i.m.) at two doses, and blood samples were taken to follow the time-course of HuBuChE in blood for up to 168 h after administration. In guinea pigs, the two doses of HuBuChE, 19.9 and 32.5 mg/kg, produced similar times of maximal blood concentration (T_{max} of 26.0 and 26.8 h, respectively) and similar elimination half-times ($t_{1/2}$ of 64.6 and 75.5 h, respectively). Enzyme levels were still 10-fold over baseline at 72 h. Based on these data, guinea pigs were administered 150 mg/kg of enzyme i.m. and challenged at T_{max}. Soman or VX doses were approximately 1.5, 2.0 and 2.0 × LD_{50} administered subcutaneously (s.c.) in sequence at 90–120 min apart. None of the animals displayed signs of organophosphorus (OP) anticholinesterase intoxication at any of the challenge levels, and all survived for the 14-day duration of the experiment. Similar experiments were carried out with cynomolgus monkeys to determine the pharmacokinetics of HuBuChE and its efficacy against soman. The complete survival of nearly all animals tested to date, coupled with the maximal blood concentration and half-life elimination profile obtained for HuBuChE after i.m. injection, provides strong support for the continued development of HuBuChE as a product to protect against nerve agents.

Keywords: Butyrylcholinesterase; Soman; VX; Guinea pigs; Cynomolgus monkey

1. Introduction

While successful, current treatments for acute nerve agent poisoning always result in the victim suffering a toxic insult that subsequently must be therapeutically managed. In contrast, recent efforts have focused on identifying proteins that can remain stable in circula-

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tion for long periods of time [1–3] and act as biological scavengers of OP compounds. This approach avoids the side effects associated with current antidotes [4–12] and the requirement for their rapid administration, by prophylactically inactivating (through sequestration or hydrolysis) anticholinesterase agents before they can react with the target acetylcholinesterase (AChE). The time frame for this inactivation to occur before endogenous AChE is affected is quite narrow (estimated to be approximately 2 min in humans [13]). For acute OP exposures, the scavenger function must be very rapid, irreversible and specific. Ideally, the scavenger should enjoy a long residence time in the blood stream, should be biologically innocuous in the absence of nerve agent, and should not present an antigenic challenge to the immune system. For these reasons, prime efforts to identify candidate bioscavengers have focused recently on HuBuChE. In a series of studies, Ashani and coworkers [14–16] examined the scavenger properties of FBS AChE and HuBuChE in mice, rats and rhesus monkeys with respect to several different nerve agents as well as other OP compounds. The main focus of those studies was to demonstrate that HuBuChE was capable of affording protection against OP poisoning as measured by the lack of behavioral deficits. In the final paper in this series [16] the authors reported protection by HuBuChE against a $3.3 \times LD_{50}$ dose of soman or a $2.1 \times LD_{50}$ dose of VX in rhesus monkeys. They also reported considerable protection against soman-induced behavioral deficits in a spatial discrimination task. Our current efforts were designed to expand on those observations to include pharmacokinetic studies of HuBuChE in two species, guinea pigs and cynomolgus monkeys, and then evaluate HuBuChE for efficacy against soman and VX poisoning in guinea pigs and against soman in cynomolgus monkeys at up to $5.5 \times LD_{50}$ doses of each agent in the respective species. Such studies would demonstrate that HuBuChE could confer consistent protection against OP poisoning in multiple species.

2. Experimental procedures

2.1. Animals

Male Hartley albino guinea pigs (*Cavia porcellus*) (Charles River Laboratories, Kingston, NY), weighing 300–450 g, were used. Animals were quarantined and observed for a minimum of 5 days for evidence of disease in accordance with the stipulations mandated for an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility prior to being put on study. Guinea pig ration and water were provided ad libitum.

Cynomolgus monkeys (3.0–3.5 kg) were held in isolation and observed for signs of clinical illness prior to study initiation. Fluorescent lighting with alternating light and dark cycles of approximately 12 h was provided. Animals were fed twice daily with Purina Certified Monkey Chow® biscuits and supplemented with fresh fruit or primate treats. Drinking water was available ad libitum. Personnel handling nonhuman primates (NHP) wore the appropriate personal protective equipment.

2.2. Materials

Soman (pinacolylmethyl phosphonofluoridate) and VX (*O*-ethyl-*S*-(2-isopropylaminoethyl) methylphosphonothiolate) were obtained from the Research Development and Engineering Center, Aberdeen Proving Ground, MD. They were >97% pure by ^{31}P NMR analysis. The HuBuChE was supplied as a lyophilized powder (700 U/mg) by the Division of Biochemistry at Walter Reed Army Institute of Research. It was dissolved in physiologic saline to the desired concentration immediately before use. Butyrylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemicals (St. Louis, MO).

2.3. Pharmacokinetic studies

Two doses of HuBuChE were injected intramuscularly (i.m.) in either guinea pigs or cynomolgus monkeys to determine its pharmacokinetics in blood. In guinea pigs, the two doses of HuBuChE were 19.9 and 32.5 mg/kg; in cynomolgus monkeys, the two doses of HuBuChE were 5.25 and 8.75 mg/kg. The lower dose in each species was calculated to provide protection against a $1.5 \times LD_{50}$ soman challenge, while the higher dose was calculated to provide protection against $2.5 \times LD_{50}$ of soman. HuBuChE was a lyophilized powder in 25 mg aliquots with a specific activity of 700 U/mg. It was reconstituted in physiologic saline to allow for injection volumes of 0.4 or 1.0 mL in guinea pigs and monkeys, respectively. Blood samples were taken in heparinized containers prior to the injection of HuBuChE (by toe clip from guinea pigs and from the femoral vein from cynomolgus monkeys) and at approximately 1, 2, 4, 10, 12, 24, 48, 72, 96, 120, 144, and 192 h after injection. BuChE activity in whole blood samples was measured in blood samples from three guinea pigs or three cynomolgus monkeys in each dose group as a function of time after administration using a microtiter plate modifica-

tion of the method of Ellman et al. [17]. Butyrylthiocholine was used as the assay substrate, and colorimetric (A_{412} nm) responses were detected using a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA).

Mean whole blood HuBuChE time–concentration data were fit to standard pharmacokinetic models using WinNonlin nonlinear regression software (version 1.5, 1997, Scientific Consulting Inc., Cary, NC). Pharmacokinetic parameter estimates and predicted HuBuChE concentrations as a function of time were calculated from the raw data using the appropriate mathematical model. The following criteria were utilized as guidelines for determining the appropriate model: minimal sum of squared residuals, high correlation coefficient, small standard deviations of parameter estimates and unbiased distribution patterns of residuals for estimates of observed versus predicted values. Parameter estimates generated were apparent volume of distribution (V_d), absorption rate constant (k_{01}), elimination rate constant (k_{10}), time to maximum plasma concentration (T_{max}), maximum plasma concentration (C_{max}), and area under the time–concentration curve (AUC).

The pharmacokinetics of HuBuChE after i.m. administration to guinea pigs or cynomolgus monkeys were best described by a one-compartment model with first-order absorption and elimination described by Eq. (1) (below):

$$C(t) = \frac{D}{V_d}\frac{k_{01}}{k_{01} - k_{10}}(e^{-k_{10}t} - e^{-k_{01}t}) \quad (1)$$

where C is the plasma concentration (mg/mL), t the time (min), D the dose (mg/kg), V_d the volume of distribution (mL/kg), k_{01} (min^{-1}), and k_{10} (min^{-1}).

2.4. Efficacy studies

Guinea pigs ($n = 10$) were administered HuBuChE i.m., in sufficient quantity to neutralize a 10–11 $\times$ LD_{50} challenge of soman (1 $\times$ LD_{50} = 30 μg/kg s.c.) or VX (1 $\times$ LD_{50} = 9 μg/kg s.c.) based on the stoichiometric equivalence of 1:1 for nerve agent and HuBuChE. At 19 (±1.0) h after administration, a blood sample was taken via toe clip and the whole blood butyrylcholinestrase (BuChE) concentration was determined. Based on that determination, the total number of moles of BuChE in circulation (endogenous and administered HuBuChE) was calculated assuming a blood volume of 20 mL for a 400 g guinea pig. A molar amount of soman (45 μg/kg) or VX (13.5 μg/kg) equal to 1.5 $\times$ LD_{50} was given s.c. and the animal observed for signs of intoxication for 90 min. At the end of that period a second blood sample was taken and the total blood BuChE concentration redetermined. The total number of moles of BuChE in circulation was recalculated, and 2 $\times$ LD_{50} of soman or VX was administered s.c. Again the animal was observed for signs of intoxication for 90 min, and if none were observed the process was repeated one more time. Ninety minutes after the third dose of soman or VX, a final blood sample was taken and analyzed for total blood BuChE concentration. Surviving animals were held for 7 days at which time one-half the surviving population was randomly selected for euthanization followed by histopathology studies. Seven days later (14 days after nerve agent administration) the remaining animals were euthanized for histopathology studies (Fig. 1).

Monkeys were administered HuBuChE, i.m., in sufficient quantity to neutralize an 8 $\times$ LD_{50} challenge of soman ($n = 6$; 1 $\times$ LD_{50} = 7 μg/kg i.m., BuChE = 10.6 mg/kg to protect against 3 $\times$ LD_{50} of soman) based on calculations that assumed a one to one stoichiometric reaction (Fig. 1). At 1.5 h pre-T_{max} after administration of enzyme, a blood sample was taken via the femoral vein, and the whole blood (BuChE) concentration was determined. Based on that determination, the total number of moles of BuChE (endogenous and administered HuBuChE) in circulation was calculated assuming a blood volume of 200 mL for a 3.0–3.5 kg monkey. An amount of soman (10.5 μg/kg) equal to

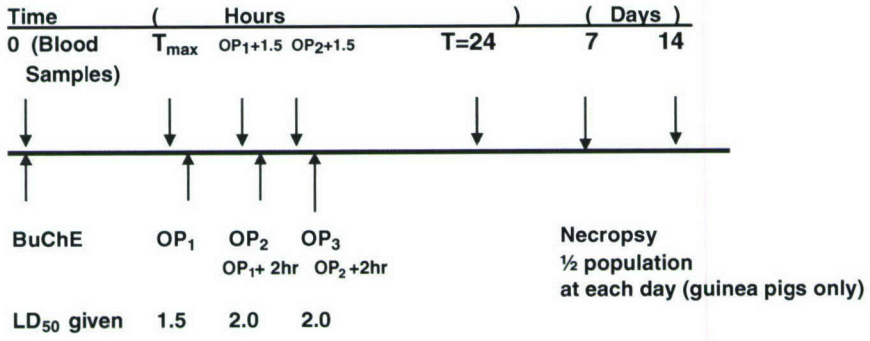

Fig. 1. Design of efficacy experiments in guinea pigs and cynomolgus monkeys.

1.5 × LD_{50} was given i.m. and the animal observed for signs of intoxication for 90 min. At the end of that period a second blood sample was taken, and the whole blood BuChE concentration redetermined. The total number of moles of BuChE in circulation was recalculated and 2 × LD_{50} of soman (14 μg/kg) was administered i.m. Again the animal was observed for signs of intoxication for 90 min, and if none were observed the process was repeated one more time. Twenty-four hours after the third dose of soman, a final blood sample was taken and analyzed for moles of HuBuChE.

3. Results

The pharmacokinetics of HuBuChE in guinea pigs followed a one-compartment model with a single elimination phase (Table 1, Fig. 2).

The pharmacokinetics of HuBuChE in cynomolgus monkeys also fit a one-compartment model with a single elimination phase (Table 2, Fig. 3).

Guinea pigs were pretreated with HuBuChE and 18–20 h later they were challenged with either soman or VX. The challenge design allowed for multiple challenges of experimental animals with lethal amounts of soman or VX until a total 5.5 × LD_{50} dose was given. This approach insured that the challenge with soman or VX occurred just prior to T_{max}. In the case of soman or VX challenge (n = 10/group) all guinea pigs survived with no observable signs (Table 3). At necropsy, either 7 days (n = 5) or 14 days (n = 5) post-soman challenge, all organs appeared normal and no abnormal histopathology was observed in Hematoxylin 2 and Eosin-Y (H&E) stained tissues. None of the animals in either of the challenge group received therapy in the form of atropine, 2-PAM or diazepam.

Monkeys were pretreated with HuBuChE, and 10–11 h later they were challenged with soman. The

Table 1
Pharmacokinetic parameters for HuBuChE in guinea pigs after i.m. administration

Parameter[a]	19.9 mg/kg HuBuChE	32.5 mg/kg HuBuChE
C_{max} (mg/mL)	0.015 ± 0.0005	0.030 ± 0.0008
T_{max} (h)	26.0 ± 2.55	26.8 ± 2.32
AUC	1.93 ± 0.19	3.84 ± 0.33
$T_{1/2}$ elimination (h)	64.6 ± 10.3	75.5 ± 10.1

[a] Values for each pharmacokinetic parameter are expressed as mean ± S.D. error (n = 3).

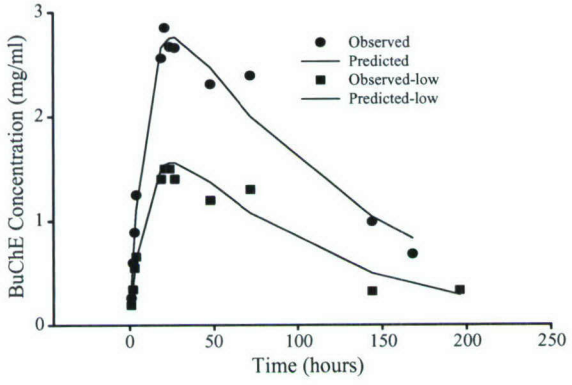

Fig. 2. Time-course of HuBuChE in blood after i.m. administration in guinea pigs: (●) 32.5 mg/kg dose of HuBuChE; (■) 19.9 mg/kg dose of HuBuChE.

Table 2
Pharmacokinetic parameters for HuBuChE in cynomolgus monkeys after i.m. administration

Parameter[a]	5.25 mg/kg HuBuChE	8.75 mg/kg HuBuChE
C_{max} (mg/mL)	0.030 ± 0.005	0.047 ± 0.0008
T_{max} (h)	9.27 ± 0.44	10.3 ± 0.47
AUC	3.68 ± 0.13	5.46 ± 0.19
$T_{1/2}$ elimination (h)	79.3 ± 3.8	73.5 ± 3.5

[a] Values for each pharmacokinetic parameter are expressed as mean ± S.D. error (n = 3).

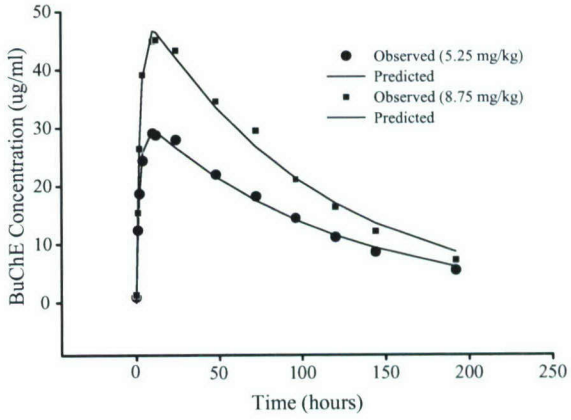

Fig. 3. Time-course of HuBuChE in blood after i.m. administration in cynomolgus monkeys: (●) 5.25 mg/kg dose of HuBuChE; (■) 8.75 mg/kg dose of HuBuChE.

Table 3
Protection by HuBuChE against nerve agent poisoning

Species	Toxin	Dose (LD_{50})	Impairment	Recovery
Guinea pig	SOMAN	5.5	None	Immediate
Guinea pig	VX	5.5	None	Immediate
Cynomolgus	SOMAN	5.5	1/5	4/6[a]

[a] One animal died after the third dose of soman and one was impaired, and later euthanized after 48 h. Remaining four animals were normal, survived and are being held for long-term observations.

challenge design allowed for multiple challenges of experimental animals with lethal amounts of soman until a total 5.5 × LD_{50} dose was given. This approach ensured that the challenge with soman occurred just prior to T_{max}. In the case of soman challenge ($n = 6$), one animal died within 30 min of receiving the third dose of soman. Of the five survivors, all lived for 48 h, but one was deemed to be in distress at that time and was euthanized after evaluation by the attending veterinarian. The other four monkeys survived with no observable signs after any of the three doses of soman. None of the tissues from the non-surviving monkeys were subjected to necropsy or histological examination.

4. Discussion

The pharmacokinetics of HuBuChE in guinea pigs were reported previously by Raveh et al. [16] following either i.m. or intravenous (i.v.) administration. In that study only a single dose of HuBuChE was administered. In the current study, we have carried out pharmacokinetic studies at two doses of HuBuChE. The lower dose, 19.9 μg/kg, is the dose of HuBuChE that would theoretically neutralize 1.5 × LD_{50} of soman, based on an LD_{50} of 30 μg/kg i.m. The higher dose, 32 mg/kg, is theoretically capable of neutralizing 2.5 × LD_{50} of soman. Given that we planned to use even higher concentrations of HuBuChE in guinea pigs for our efficacy studies, it was necessary to ensure that the pharmacokinetics of the material were not altered by changes in dose in any unexpected manner. As can be seen (Table 1), the increases in C_{max} were dose dependent in a linear manner and T_{max} was dose independent. The pharmacokinetic data following the administration of two doses of HuBuChE (5.25 mg/kg and 8.75 mg/kg equal to the theoretical amounts needed to neutralize 1.5 and 2.5 × LD_{50} of soman, respectively; 1 × LD_{50} = 8 μg/kg) to cynomolgus monkeys yield results consistent with those found in guinea pigs (Table 2). Raveh et al. had determined the pharmacokinetics for HuBuChE in rhesus monkeys at one dose and found that by 75 h, most of the material had been eliminated with a mean retention time of 34 h (following i.v. administration) and a T_{max} of ~9.5 h [16]. While the cynomolgus monkeys exhibited similar T_{max} values for maximal absorption (10.3 h), the $t_{1/2}$ of HuBuChE in this species was almost twice as long as previously reported in rhesus monkeys [16]. However, using the same preparation of HuBuChE administered i.v., a mean residence time of 72 ± 7 h was observed in rhesus monkeys (Myers et al., unpublished results). Therefore, the pharmacokinetic profile of HuBuChE in the two macaque species is very similar.

To evaluate the efficacy of HuBuChE, guinea pigs or cynomolgus monkeys were pretreated with HuBuChE at doses calculated to protect against a challenge of 8 × LD_{50} of nerve agent. This dose was chosen based on previous data (Saxena et al., unpublished results) that not all the material injected i.m. was accounted for in the circulatory system of animals. The guinea pigs or monkeys in this study were challenged three times within a 4-h period (Fig. 1). In the case of guinea pigs, the first challenge dose of soman or VX was 1.5 × LD_{50}, respectively. The second and third challenges of soman or VX were 2 × LD_{50} at 2 and 4 h, respectively, following the initial challenge for a total challenge of 5.5 × LD_{50} for each agent. The time of challenge covered a 4-h period and was designed to occur at or just prior to the T_{max} as determined in the pharmacokinetic studies. Analysis of the data used to generate the best fit model for the determination of the pharmacokinetic constants showed that the HuBuChE plasma concentrations were essentially constant at T_{max} ± 3.0 h. Based on those results, the actual challenge of guinea pigs was carried out such that the final challenge dose of soman or VX was given within the time for C_{max} (T_{max} ± 3.0 h). In the case of either soman or VX, all animals survived the entire challenge sequence, exhibiting no obvious ill effects. They were asymptomatic and did not require, nor receive, any therapeutic drugs such as atropine or 2-PAM. Necropsy results were normal and no abnormal histopathology by H&E staining was observed.

The monkeys in this study were also challenged three times within a 4-h period (Fig. 1). The first dose of soman was 1.5 × LD_{50}, The second and third challenges of soman were 2 × LD_{50} at 2 and 4 h following the initial challenge for a total challenge of 5.5 × LD_{50} for each agent. As in the guinea pig studies the actual challenge of animals was carried out such that the final challenge dose of soman was given within the time for C_{max} (T_{max} ± 3.0 h). Of the six monkeys, all survived with no indication of adverse signs after two challenges of soman, a total of 3.5 × LD_{50} in a 2-h period. After the second injection of 2 × LD_{50} of soman, one animal exhibited signs of intoxication within 10 min and died within 2 h. A second animal was lethargic and remained so for over 48 h at which time he was euthanized at the recommendation of the attending veterinarian. The remaining four animals exhibited no adverse signs. They are still alive and are being monitored for any long-term effects.

Based on our results to date, we are encouraged by the extent of protection that can be afforded by the administration of the stoichiometric scavenger, HuBuChE.

The successful results in these experiments provide the type of efficacy data in two species as required under the US Food and Drug Administration animal rule and should allow for a subsequent investigational new drug submission.

The animal care programs of the US Army Medical Research Institute of Chemical Defense and Battelle are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals proposed by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, DHHA, National Institutes of Health Publication 85-23, 1985 and the Animal Welfare Act of 1966, as amended.

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